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A functional correlate of severity in alternating hemiplegia of childhood

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ABSTRACT

Objective: Mutations in *ATP1A3*, the gene that encodes the $\alpha 3$ subunit of the Na^+/K^+ ATPase, are the primary cause of alternating hemiplegia of childhood (AHC). Correlations between different mutations and AHC severity were recently reported, with E815K identified in severe and D801N and G947R in milder cases. This study aims to explore the molecular pathological mechanisms in AHC and to identify functional correlates for mutations associated with different levels of disease severity.

Methods: Human wild type *ATP1A3*, and E815K, D801N and G947R mutants were expressed in *Xenopus laevis* oocytes and Na^+/K^+ ATPase function measured. Structural homology models of the human $\alpha 3$ subunit containing AHC mutations were created.

Results: The AHC mutations examined all showed similar levels of reduction in forward cycling. Wild type forward cycling was reduced by coexpression with any mutant, indicating dominant negative interactions. Proton transport was measured and found to be selectively impaired only in E815K. Homology modeling showed that D801 and G947 lie within or near known cation binding sites while E815 is more distal. Despite its effect on proton transport, E815K was also distant from the proposed proton transport route.

Interpretation: Loss of forward cycling and dominant negativity are common and likely necessary pathomechanisms for AHC. In addition, loss of proton transport correlated with severity of AHC. D801N and G947R are likely to directly disrupt normal Na^+/K^+ binding while E815K may disrupt forward cycling and proton transport via allosteric mechanisms yet to be elucidated.

Keywords: Na^+/K^+ ATPase; Alternating hemiplegia of childhood; *ATP1A3*

INTRODUCTION

Alternating hemiplegia of childhood (AHC) is a neurological disorder characterized by repeated attacks of paralysis on one or both sides of the body beginning before 18 months of age. There is a pressing need for effective AHC treatment. Flunarizine, a non-selective calcium channel blocker, is a widely prescribed drug for AHC. Unfortunately, flunarizine only provides symptomatic relief and its efficacy varies between patients (Mikati et al., 2000; Neville and Ninan, 2007; Sweney et al., 2009). The development of AHC therapeutics has been hindered by its complex clinical presentation. There is a well recognized clinical heterogeneity in AHC, where some patients have longer or more frequent hemiplegic attacks in addition to greater extent of cognitive dysfunction and presence of co-morbidities such as seizure and respiratory complications. In contrast, some patients have relatively mild developmental delay and with few or no co-morbidities (Mikati et al., 2000; Sasaki et al., 2014; Yang et al., 2014).

Sequencing studies identified mutations in the gene, *ATPIA3*, as a primary cause of AHC (Heinzen et al., 2012; Ishii et al., 2013; Rosewich et al., 2012). Three recurring mutations within the gene account for ~60% of all AHC cases, D801N, E815K, and G947R. Furthermore, genotype-phenotype analysis revealed that these mutations correlated with clinical severity. In general, patients with D801N or G947R have better clinical outcomes than patients with E815K (Sasaki et al., 2014; Yang et al., 2014). Because the genetic correlation with disease severity is strong, environmental factors are unlikely to play a major role in determining severity. The molecular and functional mechanisms responsible for this clinical heterogeneity are unknown.

ATPIA3 encodes for the $\alpha 3$ subunit of the Na^+/K^+ ATPase. The $\alpha 3$ subunit is neuron specific, and is highly expressed in the cortex, hippocampus, basal ganglia and thalamus (McGrail et al., 1991). The $\alpha 3$ subunit has 10 transmembrane α -helices which contain the Na^+ and K^+ binding sites and the cytoplasmic domains involved in ATP hydrolysis (Bublitz et al., 2010). The majority of AHC mutations identified are located within the transmembrane helices (>70%) (Heinzen et al., 2012). Na^+/K^+ ATPase critically regulates the Na^+ and K^+ electrochemical gradients via forward cycling. Forward cycling describes the process by which Na^+/K^+ ATPase uses ATP hydrolysis to transport three Na^+ out and two K^+ into the cell (Post et al., 1972). Recently, the Na^+/K^+ ATPase has also been shown to conduct protons under physiologically relevant conditions. It is proposed that while Na^+ ions are leaving the Na^+/K^+ ATPase during forward cycling, an aqueous path is exposed, which allows protons to passively enter the cell (Vedovato and Gadsby, 2014). This newly revealed function is well positioned to impact neuronal excitability on account of well documented effects of

intracellular protons on ion channels and receptors (Church et al., 1998; Takahashi and Copenhagen, 1996; Tombaugh and Somjen, 1996; Traynelis and Cull-Candy, 1991; Waldmann and Lazdunski, 1998).

The functional impact of *ATPIA3* mutations on Na^+/K^+ ATPase have been examined in model systems. Protein blots showed that mutations do not alter $\alpha 3$ subunit membrane expression while enzymatic assays found significant reductions in ATPase and phosphorylation activities, critical steps for proper forward cycling (Heinzen et al., 2012; Weigand et al., 2014). However, the extent of reduction in ATPase and phosphorylation activity was similar between mutations associated with mild and severe AHC. The binding capacity to ouabain, a Na^+/K^+ ATPase inhibitor, was also examined. Although D801N showed normal ouabain binding capacity it was absent in G947R and E815K (Weigand et al., 2014) and, importantly, no correlation with the disease severity was observed.

On the strength of genetic findings, this study hypothesized that the biophysical changes caused by individual AHC mutations are responsible for the correlations with AHC severity. Human mutations D801N, G947R and E815K, were expressed in *Xenopus laevis* oocytes and examined using electrophysiological techniques. The properties examined were forward cycling, dominant negativity and proton transport. Homology models of the human $\alpha 3$ subunit were also created to predict the structural-functional impact of mutations. A better understanding of *ATPIA3* mutations implicated in AHC may improve clinical diagnosis and prognosis and also revealing novel therapeutic approaches.

MATERIALS AND METHODS

Plasmid preparation

The *ATP1A3* mutations examined were: c.2401G>A (D801N), c.2839G>C (G947R) and c.2443G>A (E815K). The *Xenopus laevis atp1b3* was synthesized by Genscript (Piscataway, NJ). Since *Xenopus laevis* oocytes have endogenous *atp1b3*, *Xenopus laevis atp1b3* was to used avoid creating additional heterogeneity of assembled Na^+/K^+ ATPases which would have reduced the power to discriminate between various genotypes. All coding sequences were subcloned into an oocyte high expression vector (Liman et al., 1992) between restriction sites *AccI* and *Bpu10I* for *ATP1A3* and *HindIII* and *BamHI* for *atp1b3*. To minimize endogenous oocyte Na^+/K^+ ATPase activity, mutations which were known to reduce ouabain sensitivity, Q108R and N119D (Jewell and Lingrel, 1991), were introduced to the human $\alpha 3$ subunit by site directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies, Santa-Clara, CA). Na^+/K^+ ATPase with reduced ouabain sensitivity can inhibited by high ouabain concentration (10 mM), but not by low ouabain concentration (10 μM) (Vedovato and Gadsby, 2010). cDNAs were transcribed into capped cRNA *in vitro* (mMessage mMachine, Ambion, Austin, TX).

Oocyte preparation and injection

Mature oocytes (Stage V or VI) were obtained from *Xenopus laevis*. Oocytes were defolliculated and isolated as previously described (Petrou et al., 1997). Oocytes were injected on the automated Roboinject platform (Multichannel Systems, Reutlingen, Germany). Each oocyte was injected with 15 to 30 ng of cRNA encoding for the human $\alpha 3$ subunit and 10 ng of cRNA encoding for the *Xenopus laevis* $\beta 3$ subunit. Total injection volume was 50 to 100 nl. Oocytes were stored in ND96 solution at 17 °C for 3 days before recording.

Two electrode voltage clamp recording

Before recording, oocytes were incubated in loading solution for 2 hours at 17 °C to increase intracellular Na^+ . The loading solution contained: (mM) 95 NaOH, 90 sulfamic acid, 5 HEPES, 10 TEA-Cl and 0.1 EGTA, pH 7.5. Two electrode voltage clamp recording was performed on the automated Roboocyte2 platform (Multichannel Systems, Reutlingen, Germany). Oocytes were impaled with electrodes that contained 1.5 M K-acetate and 0.5 M KCl and were held at -50 mV. Voltage dependence was determined by measuring current in a series of 400 ms step from -140 to

+40 mV every 2 s in 20 mV increments. Recording solution used to measure forward cycling contained: (mM) 115 NaOH, 110 sulfamic acid, 10 HEPES, 5 BaCl₂, 1 MgCl₂ and 0.5 CaCl₂, pH 7.5, as well as 10 μM ouabain to inhibit endogenous oocyte Na⁺/K⁺ ATPase. Forward cycling was activated by 15 mM of K-sulfamate. Recording solution used to measure proton transport was Na⁺ and K⁺ free, the NaOH was replaced with an equal concentration of TMA-OH. Responses were quantified at the end of the test pulse. All responses were subtracted from the background to ensure responses observed were from exogenous Na⁺/K⁺ ATPase. The background response was determined by adding 10 mM ouabain to recording solution, which would inhibit all Na⁺/K⁺ ATPase, including the ouabain insensitive Na⁺/K⁺ ATPase (Poulsen et al., 2010; Vedovato and Gadsby, 2010). Sampling frequency was 1 kHz and recording temperature was 20-22 °C.

Homology modeling

Models of Na⁺ and K⁺ bound states of the human α3 subunit of the Na⁺/K⁺ ATPase were made using the structure of Na⁺/K⁺ ATPases from Pig Kidney (Kanai et al., 2013) (PDB accession code 3WGU) and shark rectal glands (Shinoda et al., 2009) (PDB accession code 2ZXE), sharing 88 and 86 % sequence identity to the human transporter respectively. Homology models were created by first aligning the sequences using Clustal W (Larkin et al., 2007), and using Modeller (Eswar et al., 2006) to generate and refine the structural model.

Data analysis

Data was exported using the Roboocyte2+ software (Multichannel Systems, Reutlingen, Germany) and analyzed using AxoGraph (AxoGraph Scientific, Sydney, Australia). Statistical analysis was performed on GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Student's t-test and one-way ANOVA were used to test for statistical significance. Statistical significance was set at $p < 0.05$.

RESULTS

Loss of forward cycling function with AHC mutations

Forward cycling was examined in oocytes expressing the wild type or AHC mutant constructs. Forward cycling current traces were first inspected visually and a consistent reduction in outward current amplitudes was observed in all mutations examined (Fig 1A) as compared to wild type. Maximum current response was observed at +40 mV. In comparison to wild type, forward cycling was reduced by 67, 79 and 69 % in D801N, G947R and E815K respectively (Fig 1B). Despite being associated with more severe AHC, the level of loss of forward cycling in E815K was similar to D801N and G947R, suggesting that loss of forward cycling function is unlikely to underlie the observed clinical heterogeneity in AHC. In this study, all AHC mutants were sensitive to 10 mM ouabain inhibition, which is inconsistent with the loss of ouabain binding capacity of G947R and E815K reported in previous ouabain binding assay (Weigand et al., 2014). However, significant reduction in forward cycling was observed in AHC mutants before 10 mM ouabain background subtraction (Supp Fig 1), therefore, the loss of forward cycling function observed in this study is unlikely due to reduced sensitivity to ouabain.

Figure 1. **(A)** Representative forward cycling response after background current subtraction (in 10 mM ouabain). Dotted line indicates the zero current level. Scale bar applies to all traces. **(B)** I-V graph of forward cycling after background current subtraction for wild type (n=18), D801N (n=12), G947R (n=6) and E815K (n=9). **(C)** Current responses observed at +40 mV. **p<0.01, one way ANOVA with post hoc Bonferroni's multiple comparison test.

Supp Fig 1. Maximum forward cycling response observed at +40 mV before 10 mM ouabain subtraction. Wild type (n=17), D801N (n=12), G947R (n=8) and E815K (n=8). WT vs D801N p value is 0.0549. * $p < 0.05$, ** $p < 0.01$, unpaired t-test.

AHC mutations are dominant negative

Patients with AHC are heterozygous, thus wild type and mutated Na⁺/K⁺ ATPase would co-exist, and it is possible for the mutant to inhibit wild type function through a dominant negative mechanism. To test this hypothesis, one group of oocytes were co-injected with 15 ng of wild type as a baseline and four additional groups of oocytes received additional injections of 15 ng of wild type or one of the three mutant $\alpha 3$ cRNAs. This design allowed for the determination of both increases and decreases in function as compared to the 15ng wild type group alone. For oocytes that were injected with 30 ng of wild type $\alpha 3$ RNA, the ratio was 1.63 ± 0.21 (Fig 2), indicating that although the oocyte model had additional headroom for functional expression it was not a simple doubling. For D801N, G947R and E815K the ratios were 0.48 ± 0.13 , 0.45 ± 0.08 and 0.57 ± 0.03 respectively (Fig 2). If the same 1.6 fold increase seen in the 30 ng wild type experiment is assumed, then the expected current ratios of the mutants would have been around 1 $[(1 + 0.3) * 0.8 = 1]$. The marked departure from ratio of 1 for each of the mutations is evidence for dominant negative interactions. The extent of dominant negativity was similar between D801N, G947R and E815K, therefore dominant negativity is unlikely to explain the AHC severity spectrum. Next, the mechanism underlying negative dominance was investigated using membrane expression of human $\alpha 3$ subunit in oocytes by Western blot. However, the expected bands for the human $\alpha 3$ subunit were faint and a poor signal to noise ratio precluded imaging or downstream comparisons with presumed dominant negative mutants (data not shown).

Figure 2. Ratio of maximum current to wild type (WT). WT (n=20), WT+WT (n=8), WT co-expressed with D801N (n=6), co-expressed with G947R (n=10) and co-expressed with E815K (n=9). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA with post hoc Bonferroni's test. For clarity, the statistical significance ($p < 0.001$) between WT+WT and all WT+AHC mutant groups are not shown.

Loss of proton transport in severe AHC mutation

A proton transport route was recently identified in the α subunit of Na^+/K^+ ATPase where the passive inward movement is suggested to be an additional physiological function of the Na^+/K^+ ATPase (Vedovato and Gadsby, 2014) enhancing intracellular concentrations of H^+ , a known modulator of neuronal excitability (Church et al., 1998; Takahashi and Copenhagen, 1996; Tombaugh and Somjen, 1996; Traynelis and Cull-Candy, 1991; Waldmann and Lazdunski, 1998). The proton transport of wild type and mutant Na^+/K^+ ATPase was measured in a Na^+ and K^+ free recording solution. Visual inspection of current traces found that wild type and the milder AHC mutations, D801N and G947R, had similar levels of proton current. In contrast, proton current amplitude was profoundly reduced in the mutation implicated in severe AHC, E815K (Fig 3A). Quantification of maximum proton current observed at -120 mV found that proton transport of E815K was significantly smaller than wild type, D801N and G947R (Fig 3B). Differences in proton transport between wild type, D801N and G947R were not significant. Because reduced proton transport function was only observed with the severe AHC mutation, E815K, this suggests that loss of proton transport is a correlate of severe AHC.

Figure 3. **(A)** Representative current traces of proton transport after background current subtraction (in 10 mM ouabain). Dotted line indicates the zero current level. Voltage steps shown are from -120 mV to -20 mV. Scale bar applies to all traces. **(B)** I-V graph of proton transport after background current subtraction for wild type (n=4), D801N (n=10), G947R (n=7) and E815K (n=10). **(C)** Current responses observed at -120 mV. * $p < 0.05$, ** $p < 0.01$, one way ANOVA with post hoc Bonferroni's multiple comparison test.

Homology models of AHC mutations

Homology models of the Na⁺ bound and K⁺ bound forms of the human $\alpha 3$ subunit of the Na⁺/K⁺ ATPase were generated. The models indicate that D801 lies in both the K⁺ and Na⁺ binding sites, so mutations at this site are likely to influence the binding affinity for both ions (Fig 4). G947 sits directly beside E951 which has previously been shown to be important in Na⁺ binding in modeling and mutagenesis studies (Li et al., 2006a; Li et al., 2006b; Ogawa et al., 2009). A basic residue at this position could compete with Na⁺ to decrease transport rates. The altered forward cycling and proton transport of E815K is more difficult to explain. Not only is this residue far from the ion binding sites it is also distant from Y768, D923 and E951 the only residues known to play a role in proton transport (Vedovato and Gadsby, 2014). E815 is accessible from the cytoplasm and forms part of a vestibule similar to that seen in ion channels. Therefore one possibility is that while a negatively charged residue at 815 enhances cation (H⁺, K⁺ and Na⁺) shuttling, the presence of a positive residue in the K815 mutant would produce a less favorable environment for cation movement. Although there is no direct evidence to support this claim, the fact that a cavity large enough to contain water molecules is seen in the protein models extending from E815 to E951 is certainly suggestive.

Figure 4. Homology models of the human $\alpha 3$ subunit of the Na^+/K^+ ATPase. **(A)** Model of the transmembrane portion of the protein highlighting the location of the residues examined in this study (purple) as well as those previously implicated in proton transport (green). The cytoplasm is at the top of the image and bound Na^+ are shown in yellow. Close up views of **(B)** the Na^+ binding sites and **(C)** the K^+ binding sites. **(D)** The solvent accessible surface of the protein. The front half of the protein has been hidden to reveal the buried cavity running between E815 (purple) and E951 (green).

DISCUSSION

This study aims to identify pathological mechanisms of *ATP1A3* mutations implicated in AHC and a functional basis for AHC clinical heterogeneity. AHC mutations D801N, G947R and E815K, were characterized *in vitro* by electrophysiology using human $\alpha 3$ subunits. Our data shows that all AHC mutations cause loss of forward cycling of the Na^+/K^+ ATPase. We also identified that AHC mutations are dominant negative, a novel pathomechanism in the three recurring AHC mutations. Data from this study strongly suggests that loss of forward cycling is a necessary pathological mechanism in AHC and it may be greater than initially expected on account of this dominant negativity. Furthermore, this study identifies loss of proton transport as a functional correlate of severe AHC suggesting that disruption of intracellular pH homeostasis participates in pathogenicity.

The loss of forward cycling function in AHC mutants observed here is consistent with previous results from enzymatic assays (Heinzen et al., 2012; Weigand et al., 2014). The molecular mechanism underlying loss of forward cycling function remains to be determined, but our homology models suggest that G947R may decrease Na^+ transport rate and D801N may affect both Na^+ and K^+ binding. Results from our homology models are consistent with a recent homology model of AHC mutations which indicated alterations to the cation binding region (Yang et al., 2014). Previous studies using enzymatic assays also suggested that K^+ binding affinity is reduced in the Na^+/K^+ ATPase containing AHC mutations (Heinzen et al., 2012; Weigand et al., 2014). Our study reveals an additional dominant negative mechanism that would contribute to loss of function. The precise mechanism of the dominant negative interaction is not known. There is some suggestion that α subunits can interact to form oligomeric complexes (Donnet et al., 2001) that provides a basis for a dominant negative interaction. Na^+/K^+ ATPase forward cycling critically maintains the Na^+/K^+ electrochemical gradient, and impaired forward cycling would cause an electrochemical disruption.

Data from the present study identifies loss of proton transport as a functional correlate of severe AHC. Proton transport is suggested to be an additional physiological function of the Na^+/K^+ ATPase (Vedovato and Gadsby, 2014), implicating its role in pH homeostasis and in regulating excitability. Impaired proton transport in the E815K mutant would suggest intracellular alkalosis, which may have severe consequences on neuronal function (Church et al., 1998; Takahashi and Copenhagen, 1996; Tombaugh and Somjen, 1996; Traynelis and Cull-Candy, 1991; Waldmann and Lazdunski, 1998). For example, it is known that alkalosis caused by hyperventilation is sufficient to increase seizure susceptibility in patients (Schuchmann et al., 2011). While loss of proton transport may be relevant to some co-morbidities observed in

severe AHC, the *in vivo* significance of proton transport via Na⁺/K⁺ ATPase is yet to be determined. Homology modeling of the E815K mutation in this study shows that this residue is distal from Na⁺/K⁺ binding sites and the proton transport route, however, E815 is accessible from the cytoplasm to form part of a charged region facilitating cation movement. This raises the possibility that E815 may participate in proton transport and that K815 may confer loss of forward cycling function via a different mechanism to D801N and G947R. To determine how loss of forward cycling and proton transport contribute to AHC pathology, investigations in AHC animal models such as the Myshkin mouse model are needed (Kirshenbaum et al., 2013).

ATPIA3 mutations are the primary cause of AHC, as well as two other neurological disorders, rapid onset dystonia of Parkinsonism (RDP;(Brashear et al., 2007)) and cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss (CAPOS; (Demos et al., 2014)). Currently, there is no functional characterization performed on CAPOS mutations. On the other hand, it is known that the majority of RDP mutations do not express on the membrane and would also result in loss of Na⁺/K⁺ ATPase function (Heinzen et al., 2012). Further studies are required to examine how mutations in the same gene lead to the three different neurological disorders and to discern genetic background effects. *ATPIA3* mutations D923N and G867D were identified in patients with an intermediate AHC-RDP phenotype (Brashear et al., 2007; Rosewich et al., 2014). These mutations may be useful in understanding the mechanism that differentiates RDP and AHC.

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